

In the Specification:

Please amend the specification as shown:

Please delete paragraph [0016] and replace it with the following paragraph:

[0016] Amino acid residues are referred to herein by their standard single-letter or three-letter notations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, Isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine. An amino acid sequence set forth herein, such as "DKLLM," (**SEQ ID NO: 1**) orders the amino acids from the N-terminus to the C-terminus in a left-to-right manner, unless otherwise indicated from the context. One of skill in the art will appreciate that non-natural amino acids are also useful in the present invention.

Please delete paragraph [0065] and replace it with the following paragraph:

[0065] Synthesis of the different test Asp protected test peptides was achieved using Boc chemistry to synthesize the sequence YAKYAKL-Pam (**SEQ ID NO: 2**). Following tyrosine incorporation, the resin was deprotected with TFA and FmocAsp(^tBu) was inserted. This was followed by piperidine deprotection (except for Troc), treatment with acetic anhydride, and ^tBu removal with TFA. After t-butyl removal, the resin was washed with DMF and the free carboxylic group protected on solid phase. For Troc, the resin was treated with 2,2,2-trichloroethanol (40 eq. of the resin free carboxylic acid), DMAP (0.1 eq.), NMM (1 eq.) and PyBop (1 eq.) in DMF overnight at 37 °C. For OPac, OMop and diMeOPac, the resin was reacted with the corresponding α -bromo ketones, respectively phenacyl bromide, α -bromo-propiophenone and 2-bromo-2'-

4'dimethoxyacetophenone. The same protocol was used for the three products, using an excess of 20 equivalents of the bromo-ketone over the resin free carboxylic acid with 1 equivalent of DIEA in DMF overnight at room temperature. The final products were cleaved and deprotected by HF treatment. The peptides were purified by preparative HPLC. OMop generated two diastereoisomers resolved by HPLC, only the major product was used. In general the yield of this procedure was 45%, only Troc generated 15% of the correct product.

Please delete paragraph [0068] and replace it with the following paragraph:

[0068] The purified peptide LYRAD(Mop)CSYRFL (SEQ ID NO: 3) obtained *via* ligation, was solubilized in aqueous 30% acetic acid, the solution was mixed with activated zinc powder for 30 minutes. The solution was recovered and the final product obtained by a desalting step with no further purification. Zinc powder (1 g) had previously been acid washed, as follow: 1N aqueous HCl (4X4 ml, 3 minute), H₂O (4X4 ml, 1 minute), and kept in H₂O until used (prepared daily).

Please delete paragraph [0069] and replace it with the following paragraph:

[0069] To investigate ligation at the Asp/Cys or Glu/Cys sites, Boc chemistry was used to synthesize two test peptide C-terminal thioesters, **(1)** LYRAD-thioester (SEQ ID NO: 4), and **(2)** LYRAE-thioester (SEQ ID NO: 5), wherein the side chain carboxy protecting group for Asp and Glu is cyclohexyl ester, and a free carboxyl peptide **(3)** CSYRFL (SEQ ID NO: 6). These short peptides were selected to increase the probability to detect by HPLC presence of side reactions. After HF cleavage, the thioester products presented a good HPLC analytical profile, with only one major component present for each

peptide, confirming the stability of these specific thioesters in acidic conditions. When aspartyl thioester peptide 1 was used to conduct a ligation reaction with the C-terminal peptide 2, with standard native chemical ligation conditions (pH 6.5, 1% thiophenol and 1% benzyl mercaptan) after 2 hours, the presence of two ligated products was noticed with slightly different retention times (R_t), both presenting the expected mass for the correct product. The relative ratio of the two products was 1:2, with the more hydrophobic product in excess. The same result was obtained when the ligation was conducted with the C-terminal Glutamyl thioester peptide, although with a smaller quantity of the more hydrophilic product, with a relative ratio of 1:4. The same result was obtained when the reaction was conducted at pH 6.2 and at pH 7.0. Elimination of the benzyl mercaptan and use of only 0.1% of thiophenol did not modify the results for both peptides. To correctly define the nature of the impurities, the native sequences (4) LYRADCSYRFL (SEQ ID NO: 7), (5) LYRAECSYRFL (SEQ ID NO: 8) were completely synthesized by solid phase techniques, as well as the unnatural isomers (6) LYRAD(β)CSYUFL (SEQ ID NO: 9) and (7) LYRAE(γ)CSYRFL (SEQ ID NO: 10). Comparing the R_t of these products with the ligation products it was confirmed that, both for the Asp ligation and for the Glu ligation, the two more hydrophilic compounds corresponded to the unnatural backbone isomers providing evidence that atoms of the thioester rearrange and migrate to the side chain carboxyl. Further evidence was provided from digestion with the enzyme SV8-protease. This proteolytic enzyme, selective for Asp-Xaa and Glu-Xaa, readily digested both the Glu and Asp ligation products that were more hydrophobic, but was not effective with the two impurities. The same results were obtained with the fully synthetic products, with product 6 and 7 being unaffected by the enzyme.

Please delete paragraph [0070] and replace it with the following paragraph:

[0070] To avoid this undesired side reaction, preferably a protecting group is selected for the side-chain carboxy that has the following characteristic: (i) stable to HF cleavage; (ii) stable during the ligation reaction; and (iii) easily removed after the ligation reaction, preferably under conditions that will not jeopardize the integrity of a fully deprotected protein. Most of the available protecting groups will not withstand the first requirement. The following protecting groups were selected for this experiment: 9-fluorenylmethyl ester (OFm)[al-Obeidi, F., *et al. Int J Pept Protein Res* 1990;**35**(3):215-8] and (phenylsulfonyl)ethyl ester (OPse)[Lee, Y. S., *et al. J Pept Res* October 1999;**54**(4):328-35]. Solid phase synthesis of **(8)** LYRAE(OFm)-thioester (**SEQ ID NO: 11**) and **(9)** LYRAE(OPse)-thioester (**SEQ ID NO: 12**) resulted in the correct peptides in both cases, with the OPse group somewhat superior in this experiment. The OFm group was associated with the presence of multiple impurities, specifically loss of OFm group (~20% of the crude material) [Xue, C. B., *et al. Int J Pept Protein Res* 1991;**37**(6):476-86] and the alkylation of the OFm group by Br-Z (~25% of the crude material). This problem is limited to peptides containing a tyrosine, but this side reaction has also been reported in the case of the Bzl protecting group [Robles, J., *et al. Int. J. Pept. Protein Res.* 1994; **43**(4): 359-62]. A further disadvantage of the OFm group lies in its high hydrophobicity, resulting in a particularly high R_f , a characteristic potentially problematic for peptide solubility when applied to a large peptide fragment.

Please delete paragraph [0072] and replace it with the following paragraph:

[0072] Protecting group OPse is preferred in some aspects, because it can be directly removed in the ligation media, increasing the final recovery. A series

of deprotection conditions were tested, using different pH and reagents (Table 1). OPse required less drastic conditions; it was removed in 2 hours in 0.1M Na₂CO₃, 10% BME, pH10 at 37 °C. OFm was removed in 15 minutes in presence of 10% BME, 20% DMF, 20% piperidine, pH12-13. β-mercaptoethanolamine affected the rate of deprotection. When these conditions were applied on (12) LYRAE(OPse)CSYRFL (SEQ ID NO: 13) and (13) LYRAE(OFm)CSYRFL (SEQ ID NO: 14), in both cases only the correct backbone peptide was generated, with no side reactions.

Please delete paragraph [0073] and replace it with the following paragraph:

[0073] A class of protecting groups that are stable to HF but are promptly removed in acidic conditions are represented by either the Phenacyl esters (OPac) [Stalakatos, G. C., *et al. J. Chem. Soc.* 1966; 11918] or by the 2,2,2-trichloroethyl esters (Troc) [Woodward, R. B., *et al. J. Am. Chem Soc.* 1966; **88**: 852]. The Asp derivatives of these groups were synthesized with a series of test peptides by a combination of Fmoc and Boc chemistry, with the aim to investigate the stability of these groups in the ligation conditions. Since there have been controversial reports on OPac stability [Yang, C. C., *et al. J. Am. Chem Soc.* 1976; **41**: 1032-104110; Bodanszky, M., *et al. J. Org. Chem.* 1978; **43**: 3071-3073] and with the aim of reducing potential hydrolysis, a sterically hindered version of Opac was designed by introducing an extra methyl in position 1:1-methyl-2-oxo-2-phenyl ester (OMop). On the other hand, two methoxy groups were introduced in positions 2' and 4' of the phenyl group to verify the incidence of the phenyl ring electron donating effect, generating the (2'-4'-dimethoxy)-phenacyl group (diMeOPac). The following compounds were synthesized: (14) Fmoc-D(Troc)YAKYAKL (SEQ ID NO: 15), (15) Ac-D(OPac)YAKYAKL (SEQ ID NO: 16) (16) Ac-D(OMop)YAKYAKL (SEQ ID NO: 17) and (17) Ac-D(diMeOPac)YAKYAKL (SEQ ID NO: 18). As reported in Table

2, the OMop group presented the higher stability, with a half-life in the ligation condition of 500 hours, sufficient to withstand a ligation. The diMeOPac presented higher stability compared to the OPac group, but less than half of the half-life of OMop. And Troc provided a half-life of less than 2 hours.

Please delete paragraph [0074] and replace it with the following paragraph:

[0074] BocAsp(Mop) was readily synthesized with only slight modifications on existing procedure for BocAsp(Pac) (Yang *et al. J. Am. Chem. Soc.* 41: 1032 (1976)). Briefly α -bromopropiophenone was reacted with the cesium salt of BocAsp-^tBu, both Boc and the t-Butyl ester were removed in one step with TFA, and the amine protected with Boc. No attempt was made to resolve the two diastereoisomers, since the protecting group is eliminated at the end of the ligation reaction. The synthesis of LYRAD(Mop)-sr (SEQ ID NO: 19) did not evidence any problem, and the final peptide was recovered in theoretical yield. At this level, it was not possible to detect the two different diastereoisomers derived from the Mop protection. Ligation with CSYRFL (SEQ ID NO: 6) was completed in 4 hours at pH 6.5, with generation of a unique ligated product, still possessing the Mop protection. The final product LYRAD(•)CSYRFL (SEQ ID NO: 20) was obtained by treatment of purified LYRAD(OMop)CSYRFL (SEQ ID NO: 21) with Zn/Acetic Acid for 30 minutes, confirming that introduction of the methyl did not affect the rate of reduction.